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(54) Title: TREATMENT OF HUMAN VIRAL INFECTIONS

(57) Abstract

Treatment of cells or humans carrying or infected with a virus capable of causing an immunodeficiency disease with particular lapachone compounds and compositions thereof.

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TREATMENT OF HUMAN VIRAL INFECTIONS

BACKGROUND OF THE INVENTION

The human immunodeficiency virus type 1 (HIV-1, also referred to as HTLV-III LAV or HTLV-III/LAV) and, to a lesser extent, human immunodeficiency virus type 2 (HIV-2) is the etiological agent of the acquired immune deficiency syndrome (AIDS) and related disorders. Barre-Sinoussi, et al., Science, 220:868-871 (1983); Gallo, et al., Science, 224:500-503 (1984); Levy, et al., Science, 225:840-842 (1984); Popovic, et al., Science, 224:497-500 (1984); Sarngadharan, et al., Science, 224:506-508 (1984); Siegal, et al., N. Engl. J. Med., 305:1439-1444 (1981); Clavel, F., AIDS, 1:135-140. This disease is characterized by a long asymptomatic period followed by the progressive degeneration of the immune system and the central nervous system. Studies of the virus indicate that replication is highly regulated, and both latent and lytic infection of the CD4 positive helper subset of T-lymphocytes occur in tissue culture. Zagury, et al., Science, 231:850-853 (1986). The expression of the virus in infected patients also appears to be regulated as the titer of infectious virus remains low throughout the course of the disease. Both HIV-1 and 2 share a similar structural and function genomic organization, having regulatory genes such as tat, rev, nef, in addition to structural genes such as env, gag and pol.

While AIDS, itself, does not necessarily cause death, in many individuals the immune system is so severely depressed that various other diseases (secondary infections or unusual tumors)

Epstein-Barr virus related lymphomas among others occur, which ultimately results in death. These secondary infections may be treated using other medications. However, such treatment can be adversely affected by the weakened immune system. Some humans infected with the AIDS virus seem to live many years with little or no symptoms, but appear to have persistent infections. Another group of humans suffers mild immune system depression with various symptoms such as weight loss, malaise, fever and swollen lymph nodes. These syndromes have been called persistent generalized lymphadenopathy syndrome (PGL) and AIDS related complex (ARC) and may or may not develop into AIDS. In all cases, those infected with the HIV are believed to be persistently infective to others.

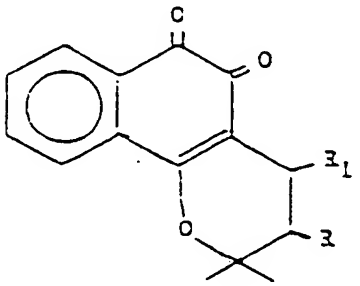
The activation of the latent HIV provirus from the asymptomatic period has been reported to be governed by long terminal repeat (LTR) in the viral DNA. See Ranki, A., et al., Lancet ii: 589-593 (1987); Fauci, A.S., et al., Science, 239:617-622 (1988); Zagury, D., et al., Science, 231:850-853 (1985); Mosca, J.D., Nature (London), 325:67-70 (1987). The activity of HIV-1 is determined by the complex interaction of positive and negative transcriptional regulators that bind to specific sequences within the LTR. Cullen, B.R., et al., Cell, 58:423-426 (1989). Changes in the quantity or quality of these factors may underlie the activation of transcription of HIV-1 and HIV-2 latent provirus by a myriad of stimuli. See Fauci, A.S., Science, 239:617-622 (1988); Griffin, G.E., et al., Nature (London), 339:70-73 (1989); Nabel, G., et al., Science, 239:1299-1302 (1988). Specifically, phorbol 12-myristate-13-acetate (PMA) and Tumor Necrosis Factor-α (TNFα) are believed to be potent activators. In particular, TNFα is present in markedly enhanced levels in HIV infected individuals, suggesting that the cytokine plays an important role in the pathogenesis of AIDS. Lahdevirta, J., Am. J. Med., 85:289-291 (1988).

Most known methods for treating individuals infected by HIV have focused on preventing integration of the virus into the host cell's chromosome or on stages other than provirus. Thus, one area of interest has been drugs that affect reverse transcriptase. Many of the proposed therapeutic methods, however, have not proven clinically effective. Indeed, even treatments that have resulted in clinical utility such as AZT (zidovudine) have not been reported to prevent the breakdown of the immune system in many patients after a number of years of treatment. Few methods have been reported to inhibit expression of the integrated provirus and chronic infection by HIV-1. Reverse transcriptase inhibitors e.g., AZT, ddc, ddI are not reported as having an inhibitory effect on chronic infections. Ro3-3335 was reported to be effective on chronic infection. See Hsu, M-C, Science 254, 1799-1802 (1992).

It thus would be desirable to have a new compound that can inhibit expression of provirus of HIV in HIV infected cells and inhibit chronic infections. It would be particularly desirable to have a new therapy that can be used to treat cells already infected, by means of inhibiting expression of provirus, or keep provirus dormant within infected cells.

SUMMARY OF THE INVENTION

We have now discovered that compounds of the following formula.I are inhibitory on expression of provirus of HIV-1 and thus are useful for treating cells that have been acutely or chronically infected by immunodeficiency viruses, such as HIV:



I

wherein R and R_1 are each independently selected from the group consisting of hydrogen, substituted and unsubstituted alkyl, substituted and unsubstituted alkenyl, substituted and unsubstituted aryl, and substituted and unsubstituted alkoxy. Preferred compounds of formula I include those in which at least one of the substituents R and R_1 is hydrogen and/or at least one of said substituents is allyl. Specifically preferred compounds include β-lapachone (i.e., R and R_1 both being hydrogen), and allyl-β-lapachone, particularly 3-allyl-β-lapachone (i.e., R being allyl and R_1 being hydrogen).

The compounds of formula I can reduce or inhibit expression of genes operably linked to the HIV LTR.

In one embodiment, the compounds of formula I can treat cells including human cells infected acutely and chronically by immunodeficiency viruses, for example HIV, preferably HIV-1, and thus can be used to treat humans infected by HIV. For example, treatment of those diagnosed as having AIDS as well as those having ARC, PGL and those not yet exhibiting such conditions.

These compounds can be used against a different target than the conventional drugs being used to treat humans infected by HIV, e.g., reverse transcriptase inhibitors such as zidovudine (AZT), 2',3'-dideoxyinosine (ddI) and 2',3'-dideoxycytidine (ddC). Using such drugs in combination with the present compounds is anticipated to result in a synergistic result. Similarly, the present compounds should be effective in cells that are resistant to such compounds. For example, compounds of the present invention can be used to block HIV-1 LTR directed expression in AZT resistant cell lines.

The invention also provides pharmaceutical compositions comprising a compound of formula I and a suitable carrier therefor for use in the conditions referred to above.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the inhibitory effects of β-lapachone (varying concentrations) on TNFα and PMA induced HIV LTR directed gene expression.

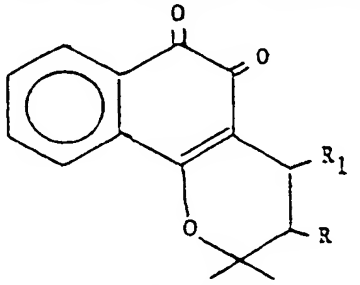
Figure 2 shows kinetics of inhibition on HIV LTR directed cytokine stimulated gene expression by β-lapachone.

Figure 3 shows inhibitory effects of 3-allyl-β-lapachone on HIV LTR directed gene expression.

Figure 4 shows inhibition of HIV-1 replication by β-lapachone in acutely infected human peripheral blood mononuclear cells.

DETAILED DESCRIPTION OF THE INVENTION

We have discovered that compounds of the following formula I can be used to treat cells infected by an immunodeficiency virus, preferably human cells infected with HIV, and thus can be used for treatment in HIV infected individuals:



I

wherein R and R_1 are each independently selected from the group consisting of hydrogen, substituted and unsubstituted aryl, substituted and unsubstituted alkenyl, substituted and unsubstituted alkyl and substituted and unsubstituted alkoxy. The alkyl groups preferably have from 1 to about 15 carbon

atoms, more preferably from 1 to about 10 carbon atoms, still more preferably from 1 to about 6 carbon atoms. As used herein, the term alkyl unless otherwise modified refers to both cyclic and noncyclic groups, although of course cyclic groups will comprise at least three carbon ring members. Straight or branched chain noncyclic alkyl groups are generally more preferred than cyclic groups. Straight chain alkyl groups are generally more preferred than branched. The alkenyl groups preferably have from 2 to about 15 carbon atoms, more preferably from 2 to about 10 carbon atoms, still more preferably from 2 to about 6 carbon atoms. Especially preferred alkenyl groups have 3 carbon atoms (i.e., 1-propenyl or 2-propenyl), with the allyl moiety being particularly preferred. Phenyl and napthyl are generally preferred aryl groups. Alkoxy groups include those alkoxy groups having one or more oxygen linkage and preferably have from 1 to 15 carbon atoms, more preferably from 1 to about 6 carbon atoms. Said substituted R and R_1 groups may be substituted at one or more available positions by one or more suitable groups such as, for example, alkyl groups such as alkyl groups having from 1 to 10 carbon atoms or from 1 to 6 carbon atoms, alkenyl groups such as alkenyl groups having from 2 to 10 carbon atoms or 2 to 6 carbon atoms, aryl groups having from six to ten carbon atoms, halogen such as fluoro, chloro and bromo, and N, O and S, including heteroalkyl, e.g., heteroalkyl having one or more of said hetero atom linkages (and thus including alkoxy, aminolakyl and thioalkyl) and from 1 to 10 carbon atoms or from 1 to 6 carbon atoms.

It is believed that the compounds of formula I provide effective therapy of chronically infected cells (i.e. cells infected by a virus which is an immunodeficiency virus such as FIV, SIV, HIV, etc.) as evidenced by a reduction in, preferably a complete repression of, HIV LTR directed gene expression. Thus, in an HIV infected cell addition of an effective amount of a compound of formula I will reduce the expression of a gene

operably linked to the HIV LTR. Preferably the gene is operably linked to an HIV-1 LTR. As used herein, the term operably linked means that the gene is under the control of the HIV LTR and positioned in a nucleotide sequence to accomplish this. Typically, the gene is downstream of the LTR, which acts as a promoter. Preferably, the gene corresponds to a viral gene such as the HIV env gene, HIV tat gene, HIV rev gene, etc.

Hence, in one preferred embodiment the present invention can be used in treating those diagnosed as having AIDS as well as those having ARC, PGL and those seropositive but asymptomatic patients. For example, as a preventative, it can also be used prophylactically as a preventative for high risk individuals.

Compounds of formula I can be used to treat cells, especially mammalian cells and in particular human cells, infected by an immunodeficiency virus such as HIV infected cells. As a result of treatment with compounds of formula I viral expression is significantly reduced. β-lapachone and allyl-β-lapachone are preferred compounds of formula I.

For example, HIV viral expression can be studied by a number of methods such as looking at the expression of a marker gene, e.g. CAT, Lac Z, etc., operably linked to the HIV LTR, which acts as the promoter. Use of the present compounds such as β-lapachone can significantly reduce expression of such a marker. HIV viral expression is turned on and enhanced by HIV LTR stimulators such as tumor necrosis factor-α (TNFα) or phorbol 12-myristate-13-acetate (PMA). One product of this expression, i.e., tat can further augment such viral expression. Using a marker gene such as Lac Z operably-linked to the HIV LTR in HIV infected cells, the addition of an effective amount of compounds of formula I significantly inhibits expression of this gene product, thereby indicating that HIV expression under the control of the HIV LTR such as HIV

envelope glycoprotein expression has been inhibited if not completely stopped.

P^{24}, a major structural protein (product of gag), has been widely used for monitoring HIV-1 replication in cells and vireamia in individuals. Use of the present compounds such as β-lapachone, at concentrations that do not significantly adversely affect cells, can dramatically reduce HIV-1 replication, e.g., preferably a reduction of more than 25% as determined by P^{24} levels, more preferably a reduction of more than 50%, and still more preferably a reduction of HIV-1 replication of more than 80% as determined by P^{24} levels.

The effective amount used to obtain such a result is at or below micromolar concentrations. Furthermore, the administration of the compounds of the present invention at effective concentrations, which inhibit HIV expression, has not been found to adversely affect cells.

The compounds of the present invention can be administered to HIV infected individuals or to individuals at high risk for HIV infection. For example, those having sexual relations with an HIV infected partner, intravenous drug users, etc. Because of its inhibitory effect, the compounds of the present invention, or pharmaceutical compositions comprising one ore more compounds of formula I can be used prophylactically as a method of prevention for such individuals to minimize their risk. One would administer the compound at an effective amount as set forth below by methodology such as described herein.

Compounds of formula I can readily be made or obtained. (See Pardee, A., et al., Cancer Research, 49, 1-8 (1989)); Schaffner-Sabba, K., et al., Journal of Medicinal Chemistry, 27, no. 8 990-994 (1984); S. Hooker, 58, 1181-1197 (1936). Other Thus, it is believed compounds of formula I will have utility in

inhibiting the progression of an HIV infection and other retroviral infections in cells and in a human, including utility in extending the latency of an HIV infection in humans.

While not wishing to be bound by theory, the absence of cytotoxicity of the compounds of formula I indicates that these compounds affect positive or negative regulators of HIV LTRs, preferably HIV-1 LTR, that are more critical to the retrovirus than the host cell.

Preferably, for inhibiting or reducing the expression of genes operably linked to an HIV LTR, one or more compounds of the invention is administered in an amount sufficient to reduce of the amount of protein expressed by the gene by at least about 25 percent relative to an untreated cell, more preferably an amount sufficient to reduce the amount of protein by at least about 50 percent and still more preferably a reduction of the amount of protein expressed by least about 75 percent relative to an untreated cell.

In general for the treatment of immunodeficiency viral infections, for example an HIV infection, a suitable effective dose of one or more compounds of formula I will be preferably in the range 1 to 5,000 μg per kilogram body weight of recipient per day, more preferably in the range of 10 to 4,000 μg per kilogram body weight per day. The desired dose is suitably administered once or several more sub-doses administered at appropriate intervals throughout the day, or other appropriate schedule. These sub-doses may be administered as unit dosage forms, for example, containing 1 to 2,000 μg, preferably 10 to 1,000 μg per unit dosage form.

Administration of the compounds of the invention may be by any suitable route including oral, rectal, nasal, topical (including buccal and sublingual), vaginal and parenteral

(including subcutaneous, intramuscular, intravenous and intradermal) with oral or parenteral being preferred. It will be appreciated that the preferred route may vary with, for example, the condition and age of the recipient.

The administered ingredients may be used in therapy in conjunction with other medicaments such as reverse transcriptase inhibitors such as dideoxynucleosides, e.g. zidovudine (AZT), 2',3'-dideoxyinosine (ddI) and 2',3'-dideoxycytidine (ddC), TAT antagonists such as Ro 3-3335, Ro 24-7429, protease inhibitors and other agents such as 9-(2-hydroxyethoxymethyl)guanine (acyclovir), interferon, e.g., alpha-interferon, interleukin II, and phosphonoformate (Foscarnet) or in conjunction with other immune modulation agents including bone marrow or lymphocyte transplants or other medications such as levamisol or thymosin which would increase lymphocyte numbers and/or function as is appropriate. Because many of these drugs are directed to different targets, e.g., viral integration, it is anticipated that a synergistic result will be obtained by this combination.

Similarly, the present compounds may be effective when the above-described drugs are not or are no longer effective. For example, compounds of the present invention can be used in cells that are resistant to reverse transcriptase inhibitors such as AZT, ddI, and ddC. For instance, the compounds of formula (I), can be used to block HIV-1 LTR directed LTR expression in such resistant cell lines and for treatment of such resistant strains. For example, the present compounds can block HIV-1 LTR directed expression in an AZT resistant strain of HIV-1. Accordingly, the present invention can be used therapeutically in an individual as that individual develops resistance to drugs that act on different targets such as AZT, ddI, ddC, RO3-3335, etc. It is expected that the present invention can be used for treatment of HIV-1 infected individuals who develop resistance

to any drug that targets a different state in the viral life cycle than the present compounds.

While one or more compounds of formula I may be administered alone, they also may be present as part of a pharmaceutical composition. The compositions of the invention comprise at least one compound of formula I together with one or more acceptable carriers thereof and optionally other therapeutic ingredients, including those therapeutic agents discussed supra. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

The compositions include those suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration. The compositions may conveniently be presented in unit dosage form, e.g., tablets and sustained release capsules, and in liposomes and may be prepared by any methods well known in the art of pharmacy.

Such methods include the step of bringing into association the to be administered ingredients with the carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers, liposomes or finely divided solid carriers or both, and then if necessary shaping the product.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous

liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion or packed in liposomes and as a bolus, etc.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, preservative, surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein.

Compositions suitable for topical administration include lozenges comprising the ingredients in a flavored basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the ingredient to be administered in a suitable liquid carrier.

Compositions suitable for topical administration to the skin may be presented as ointments, creams, gels and pastes comprising one or more compounds of formula I and a pharmaceutically acceptable carrier. A suitable topical delivery system is a transdermal patch containing the ingredient to be administered.

Compositions suitable for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

Compositions suitable for nasal administration wherein the carrier is a solid include a coarse powder having a particle

size, for example, in the range 20 to 500 microns which is administered in the manner in which snuff is taken, i.e., by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable formulations wherein the carrier is a liquid, for administration, as for example, a nasal spray or as nasal drops, include aqueous or oily solutions of the active ingredient.

Compositions suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such carriers as are known in the art to be appropriate.

Compositions suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable for oral administration may include flavoring agents.

All documents mentioned herein are incorporated herein by reference.

The present invention is further illustrated by the following examples. These examples are provided to aid in the understanding of the invention and are not to be construed as limitations thereof.

GENERAL COMMENTS

The following reagents and procedures were employed as specified in the examples.

Virus. HIV-1 was obtained from the culture supernatant of HTLV-III_B-producing H9 (H9/HTLV-III_B) cells. During the exponential phase of growth, cell-free supernatant was harvested, standardized for reverse transcriptase (RT) activity, and frozen in aliquots at -70°C. Clinical isolates of HIV-1 were prepared from patients testing positive for the human immunodeficiency virus, and standardized for RT activity.

Cells. Cell clone 293.27.2 obtained from L.A. Herzenberg (Stanford University) was derived from human embronic kidney epithelial cells, which were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% Fetal Calf Serum (FCS, obtained from Sigma) plus L-glutamine. See Roederer, M., et al., Proc. Natl. Acad. Sci. USA, 87:4884-4888 (1990). This cell clone had been stably transfected with PNAZ, which is an expression construct of lacZ gene driven by HIV-1 LTR. Expression of β-galactosidase can be greatly increased by administration of PMA or TNFα. Human peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Hypaque gradient centrifugation of blood from HIV-seronegative individuals, and cultured in RPMI 1640 supplemented with 20% FCS (Sigma), penicillin, streptomycin, and L-glutamine in the presence of PHA (3 μg/ml). RPMI 8402 cell line, received

from Tushino Ando (Aichi Cancer Research Institute, Nagoya, Japan), is a human T lymphatic cell line. It was grown in RPMI 1640 medium supplemented with 15% FCS and L-glutamine.

Stock Solution. A stock solution was prepared of β-lapachone in dimethylsulfoxide (DMSO) at a 20 mM concentration. Aliquots of the stock solution were stored frozen at -20°C.

Quantitation of HIV-1 LTR directed gene expression. Exponentially growing 293.27.2 cells (L.A. Herzenberg of Stanford University) were plated in 6 well plates at 2 x 10^5 cells per well in 2 ml of growth medium. After 48 hours, the cells were stimulated with 40 u/ml of TNFα (Genzyme, Cambridge, MA) or 2 ng/ml of PMA (Sigma). Various concentrations of β-lapachone was added to the medium at designated times after stimulation with final concentrations of DMSO or ethanol at less than 0.1% (v/v). After 6-8 hours incubation at 37°C, the medium was aspirated, cells were harvested, washed 4 times with PBS, and lysed in lacZ buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$). β-galactosidase activities of cell lysates were quantitated by using ONPG as substrate. See, e.g. Herbomel, et al., Cell, 39:653-662 (1984). Protein concentration was measured. Cell visibility was determined by the colony formation assay after cells were treated as above.

Treatment in acute HIV-1 infection. Peripheral blood mononuclear cells (PBMC), after 72 hours stimulation with 3 μg/ml PHA, were infected with either HTLV-III or a clinical isolate of HIV-1 at 1 reverse transcriptase unit (RTU) per 10 cells. Infection was carried out at 37°C for 2 hours. Then PBMC were washed with PBS to remove free virus and replated at 4.5 X 10^6 cells per well in 2 ml medium in the absence or presence of different concentrations of drugs. The cells were

then continuously exposed to drugs for 6 days. On day 3, 1 ml of medium was removed from each well and replaced with 1 ml of fresh media containing drug at the previous concentration. On day 6, cells and media were harvested. Cell viability was determined by the trypan blue exclusion method and MTT metabolic assays. See Mosman, T., J. Immunological Methods, 65:55-63 (1983). P^{24} levels in the culture supernatant were quantitated by ELISA assay with HIV-1 P^{24} Antigen Kinetics Assay Kit (Coulter, Hialeah, FL) used according to the manufacturer's protocol. RNA of HIV-1 reverse transcriptase (RT RNA) was assayed with HIV-1 RNA Detection Kit (GeneTrak, Framingham, MA) according to manufacturer's instructions. Briefly, the total cellular RNA was prepared, dot blotted onto a nitrocellulose membrane, and hybridized with ^{32}P labeled probe for RT RNA.

EXAMPLE 1

The effects of β-lapachone on HIV LTR directed cytokine enhanced gene expression (LacZ) was examined as described below, with the results depicted in Figure 1. A model HIV provirus transfected cell line, 293.27.2 (see General Comments above), was plated in 6 well plates at 2 X 10^5 cells/well in 2 ml of growth medium (DMEM). TNFα (40 u/ml) and PMA (2 ng/ml) together with various concentrations of β-lapachone as indicated in Figure 1 were added to culture media 48 hours after the cells were plated. Six hours after addition of the drug, the cells were harvested and β-galactosidase activity was measured with ONPG as the substrate by the methodology described in Herbomel, et al., Cell, 39:653-662 (1984). Enzymatic activity was expressed as percentage of maximum expression which is referred to that in drug untreated sample (taken as 100). The experiments were repeated on three independent occasions. For cell survival analysis, 293.27.2 cells were treated similarly as above. After treatment with different concentrations of β-lapachone for 6 hours, cells were

trypsinized and replaced in triplicate at 100, 1,000, 10,000 cells per 60 mm plate. After 7 day incubation, cell cloning (more than 50 cells) were counted. Plating efficiency was around 20%. Results are depicted in Figure 1 where each data point is expressed as percent survival of control, i.e. drug untreated plates (taken as 100).

As depicted in Figure 1, β-lapachone exhibited strong inhibition of expression of HIV LTR in the absence of a decrease in cell survival. β-lapachone provided inhibition against stimulation by PMA at an IC_{50} of 0.3 μM, and inhibition against stimulation by TNFα at 0.7 μM. As shown in Figure 1, β-lapachone can reduce HIV LTR activity to near zero, indicating its inhibition of basal activity. These results suggest that the compounds of formula I inhibit LTR-activation of HIV-1 provirus.

EXAMPLE 2

Cells were plated as described in Example 1 above. TNFα (40 u/ml) was added to culture media forty eight hours after the cells were plated or PMA (2 ng/ml). β-lapachone at a concentration of 2 μM was added to culture media with TNFα. β-galactosidase was assayed as described in Example 1 above. Experiments were repeated twice independently. Results are depicted in Figure 2 where each data point represents an average of triplicate culture walls.

EXAMPLE 3

The same procedures described in Example 2 above was repeated using 3-allyl-β-lapachone in place of β-lapachone with the results depicted in Figure 3.

EXAMPLE 4

The effect on HIV-1 replication in acutely infected PBMC by β-lapachone was examined as described below with the results

depicted in Figure 4. Human PBMC were infected with HTLV-IIIB at 37°C for 2 hours. After stimulation with PHA (3 μg/ml) for 72 hours, free virus were removed by wash with PBS. Different concentrations of β-lapachone was added to the culture media in less than 0.1% (v/v) of DMSO. P^{24} levels in culture supernatant were determined as described in the General Comments above. Cell viability was ascertained by trypan blue exclusion. As shown in Figure 4, a dose dependent decline in HIV-1 replication was evident for β-lapachone in the absence of significant toxicity. The therapeutic index (defined herein as the ratio of the 50% cell growth inhibitory concentration to 50% virus inhibitory concentration) was estimated to be 5.0.

EXAMPLE 5

Inhibition of HIV-1 expression in chronically infected human T cell lines. RPMI 8402 were infected with HTLV-III. β-Lapachone was added to the culture media of the chronically infected cells (2×10^5 cell/ml). The final concentrations of DMSO in media were kept at less than 0.1%. Three and six days after addition of drugs, P^{24} levels in the culture supernatant were determined as described in the General Comments above. Cell viability was determined by MTT as described in the General Comments. Results are shown in the Table below.

TABLE

Inhibition of HIV-1 replication in chronically infected human T lymphocytes by β-Lapachone*

β-lapachone concentration (μM)	inhibition of P^{24}(%)	cell viability (%)
0	0	100
0.625	18	100
2.5	60	100

* RPMI 8402 cell line was used.

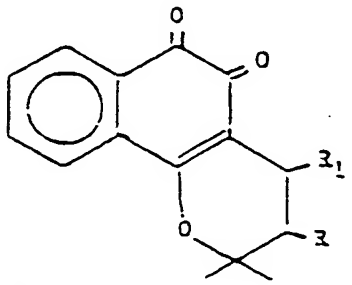
EXAMPLE 6

In a chronically infected T-lymphocyte line and the promyelocytic cell line OM10.1, inhibition of expression of P^{24} antigen was seen at IC_{50} - 0.057 μM following treatment with β-lapachone whereas AZT and ddI had little effect. The cell line OM10.1 contains one copy of HIV-1 cell integrated into its genome and continually produces a low level of HIV-1 proteins. This cell line was kindly supplied by NIH-AIDS Research and Reference Reagent Program.

This invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modification and improvements within the spirit and scope of the invention.

What is claimed is:

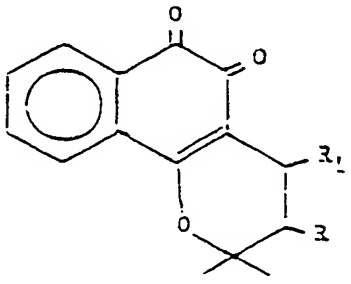
1. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an effective antiviral treatment amount a compound of the formula I:



I

wherein R and R_1 are each independently selected from the group consisting of hydrogen, substituted and unsubstituted alkyl, substituted and unsubstituted alkenyl, substituted and unsubstituted aryl, and substituted and unsubstituted alkoxy, adapted for treating a mammal having an immunodeficiency virus.

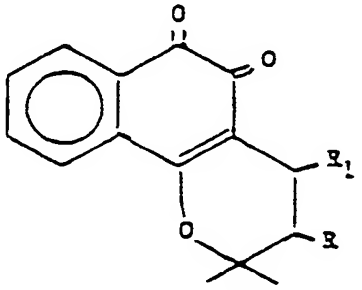
2. A method of inhibiting or reducing the expression of genes operably linked to an LTR of an immunodeficiency virus which comprises administering an effective gene expression reduction amount of a compound of the formula I:



I

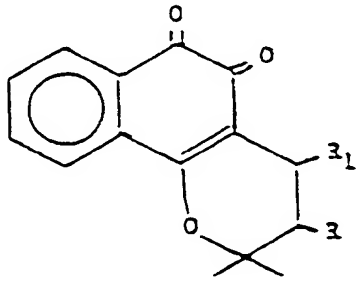
wherein R and R_1 are each independently selected from the group consisting of hydrogen, substituted and unsubstituted alkyl, substituted and unsubstituted alkenyl, substituted and unsubstituted aryl, and substituted and unsubstituted alkoxy.

3. A method for treating cells infected with a virus capable of causing an immunodeficiency disease in a human, comprising administering to the cells an effective antiviral treatment amount of a compound of the following formula I:



wherein R and R_1 are each independently selected from the group consisting of hydrogen, substituted and unsubstituted alkyl, substituted and unsubstituted alkenyl, substituted and unsubstituted aryl, and substituted and unsubstituted alkoxy.

4. A method of treating a human having an immunodeficiency disease comprising administering to said human an effective immunodeficiency disease treatment amount of a compound of the following formula I:



I

wherein R and R_1 are each independently selected from the group consisting of hydrogen, substituted and unsubstituted alkyl, substituted and unsubstituted alkenyl, substituted and unsubstituted aryl, and substituted and unsubstituted alkoxy.

5. The immunodeficiency virus of claims 1, 2, 3 or 4 which is resistant to a reverse transcriptive inhibitor.

6. The reverse transcriptive inhyibitor described in claim 5, which is selected from the group consisting of zidovudine (AZT), 2',3'-dideoxyinesine (ddI) and 2',3'-dideoxyctidine (ddC).

7. The reverse transcriptive inhibitor described in claim 6 which is AZT.

8. The method of claim 2 wherein the LTR is an HIV LTR.

9. The method of claim 2 wherein the LTR is an HIV-1 LTR.

10. The compound described in claims 1, 2, 3 or 4, where at least one of R and R_1 is hydrogen.

11. The compound described in claims 1, 2, 3 or 4, where at least one of R and R_1 is alkenyl.

12. The compound described in claims 1, 2, 3 or 4, where at least one of R and R_1 is allyl.

13. The compound described in claims 1, 2, 3 or 4 where the compound of formula I is selected from the group consisting of β-lapachone and allyl-β-lapachone.

14. The compound described in claim 13, where the compound of formula I is 3-allyl-β-lapachone.

15. The method of claim 3 where the cells are mammalian cells.

16. The method of claim 3 where the cells are human cells.

17. The immunodeficiency virus described in claims 1, 2, 3, 4 or 5 where the virus is capable of causing in the human acquired immune deficiency syndrome or an acquired immune deficiency syndrome related complex.

18. The virus described in claim 17 where the virus is HIV.

19. The virus described in claim 18 where the virus is HIV-1.

20. The method of claim 4 in which the human has antibodies to the HIV virus.

21. The method of claim 4 in which the human has antibodies to the HIV-1 virus.

FIGURE 1

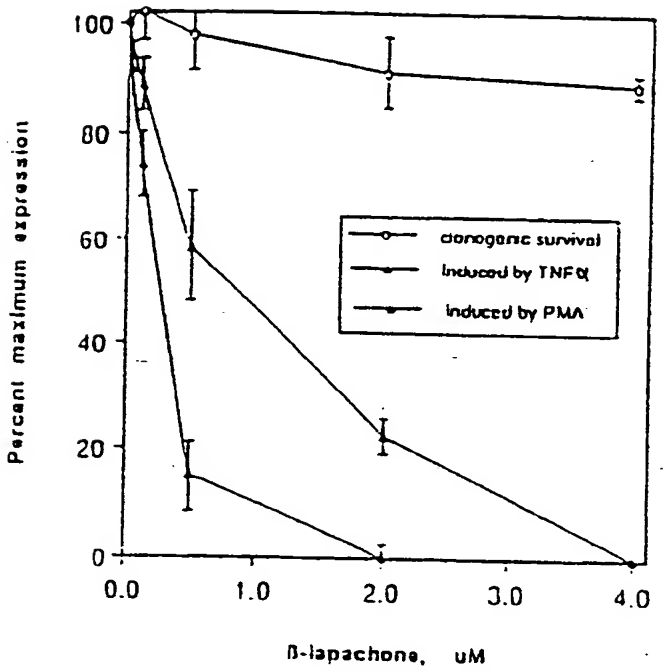
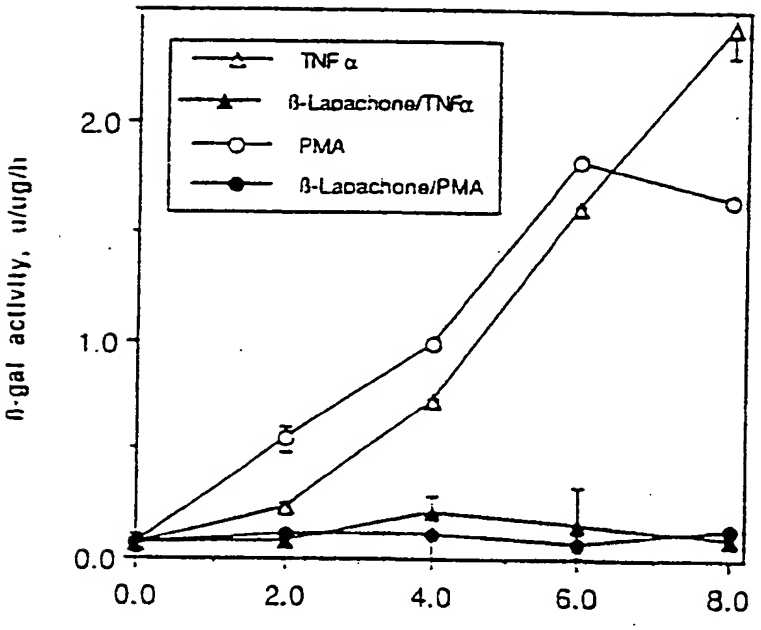
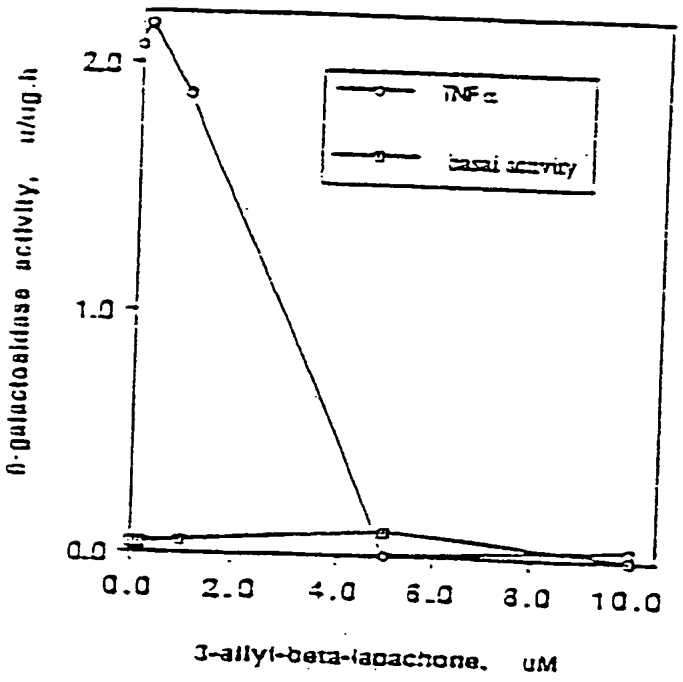

100
80
60
40
20
0
0.0
1.0
2.0
3.0
4.0
Percent maximum expression
clonogenic survival
induced by TNFα
induced by PMA
β-lapachone, uM

FIGURE 2



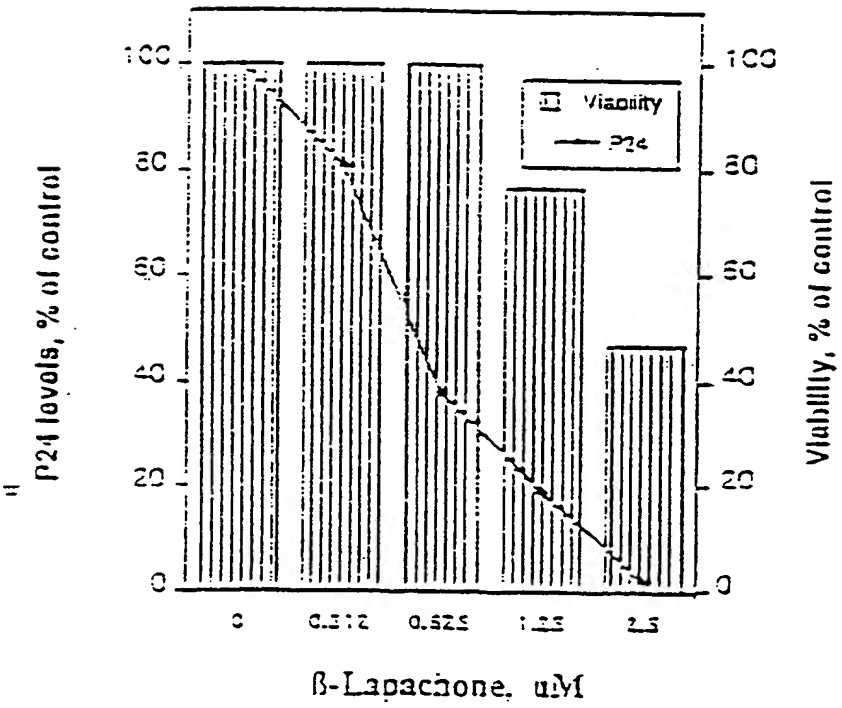
Kinetics of inhibition of beta-Lapachone on HIV LTR directed cytokine stimulated gene expression

FIGURE 3



Inhibitory effects of 3-allyl-beta-lapachone on HIV LTR directed gene expression

FIGURE 4



Inhibition of HIV-1 replication by ß-Lapachone in acutely infected human peripheral blood mononuclear cells

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US93/07878

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :A61K 31/35
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/455

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN - Structure, Antiviral methods of use AIDS, HIV

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Chemical Abstracts, Volume 101, No. 7, 13 August 1984, issued (Columbus, Ohio, U.S.A.), "Schaffner-Sabba et al, "Beta-Lapachone: synthesis and activities in tumor models". See the abstract no. 54755x. Chem. biol. Res. Lab., Ciba-Geigy Ltd., Basle, Switz.), J. Med. Chem., 1984, vol. 27, no. 8, pp. 990-994.	1-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US93/07878

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Chemical Abstracts, Volume 88, no. 20, issued 13 November 1978 (Columbus, Ohio, U.S.A.), Schurech et a, "Beta-Lapachone, an inhibitor for oncornavirus referse transcriptase and eukaryotic DNA polymerase-alpha. Inhibitory effect, thiol dependency and specificity". (Pharm. Div., Ciba-Geigy Ltd., Basel, Switz.) See abstract no. 165933, Eur. J. Biohcem. 1978, vol. 84, no. 1, pp. 197-205.	1-21

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(54) Title: TREATMENT OF HUMAN VIRAL INFECTIONS

(57) Abstract

Treatment of cells or humans carrying or infected with a virus capable of causing an immunodeficiency disease with particular lapachone compounds and compositions thereof.

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TREATMENT OF HUMAN VIRAL INFECTIONS

BACKGROUND OF THE INVENTION

The human immunodeficiency virus type 1 (HIV-1, also referred to as HTLV-III LAV or HTLV-III/LAV) and, to a lesser extent, human immunodeficiency virus type 2 (HIV-2) is the etiological agent of the acquired immune deficiency syndrome (AIDS) and related disorders. Barre-Sinoussi, et al., Science, 220:868-871 (1983); Gallo, et al., Science, 224:500-503 (1984); Levy, et al., Science, 225:840-842 (1984); Popovic, et al., Science, 224:497-500 (1984); Sarngadharan, et al., Science, 224:506-508 (1984); Siegal, et al., N. Engl. J. Med., 305:1439-1444 (1981); Clavel, F., AIDS, 1:135-140. This disease is characterized by a long asymptomatic period followed by the progressive degeneration of the immune system and the central nervous system. Studies of the virus indicate that replication is highly regulated, and both latent and lytic infection of the CD4 positive helper subset of T-lymphocytes occur in tissue culture. Zagury, et al., Science, 231:850-853 (1986). The expression of the virus in infected patients also appears to be regulated as the titer of infectious virus remains low throughout the course of the disease. Both HIV-1 and 2 share a similar structural and function genomic organization, having regulatory genes such as tat, rev, nef, in addition to structural genes such as env, gag and pol.

While AIDS, itself, does not necessarily cause death, in many individuals the immune system is so severely depressed that various other diseases (secondary infections or unusual tumors)

Epstein-Barr virus related lymphomas among others occur, which ultimately results in death. These secondary infections may be treated using other medications. However, such treatment can be adversely affected by the weakened immune system. Some humans infected with the AIDS virus seem to live many years with little or no symptoms, but appear to have persistent infections. Another group of humans suffers mild immune system depression with various symptoms such as weight loss, malaise, fever and swollen lymph nodes. These syndromes have been called persistent generalized lymphadenopathy syndrome (PGL) and AIDS related complex (ARC) and may or may not develop into AIDS. In all cases, those infected with the HIV are believed to be persistently infective to others.

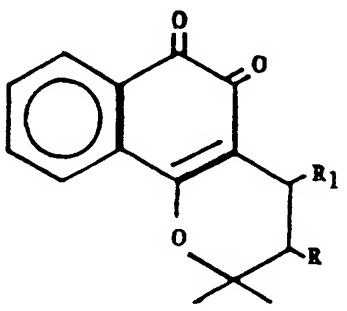
The activation of the latent HIV provirus from the asymptomatic period has been reported to be governed by long terminal repeat (LTR) in the viral DNA. See Ranki, A., et al., Lancet ii: 589-593 (1987); Fauci, A.S., et al., Science, 239:617-622 (1988); Zagury, D., et al., Science, 231:850-853 (1985); Mosca, J.D., Nature (London), 325:67-70 (1987). The activity of HIV-1 is determined by the complex interaction of positive and negative transcriptional regulators that bind to specific sequences within the LTR. Cullen, B.R., et al., Cell, 58:423-426 (1989). Changes in the quantity or quality of these factors may underlie the activation of transcription of HIV-1 and HIV-2 latent provirus by a myriad of stimuli. See Fauci, A.S., Science, 239:617-622 (1988); Griffin, G.E., et al., Nature (London), 339:70-73 (1989); Nabel, G., et al., Science, 239:1299-1302 (1988). Specifically, phorbol 12-myristate-13-acetate (PMA) and Tumor Necrosis Factor-α (TNFα) are believed to be potent activators. In particular, TNFα is present in markedly enhanced levels in HIV infected individuals, suggesting that the cytokine plays an important role in the pathogenesis of AIDS. Lahdevirta, J., Am. J. Med., 85:289-291 (1988).

Most known methods for treating individuals infected by HIV have focused on preventing integration of the virus into the host cell's chromosome or on stages other than provirus. Thus, one area of interest has been drugs that affect reverse transcriptase. Many of the proposed therapeutic methods, however, have not proven clinically effective. Indeed, even treatments that have resulted in clinical utility such as AZT (zidovudine) have not been reported to prevent the breakdown of the immune system in many patients after a number of years of treatment. Few methods have been reported to inhibit expression of the integrated provirus and chronic infection by HIV-1. Reverse transcriptase inhibitors e.g., AZT, ddc, ddI are not reported as having an inhibitory effect on chronic infections. Ro3-3335 was reported to be effective on chronic infection. See Hsu, M-C, Science 254, 1799-1802 (1992).

It thus would be desirable to have a new compound that can inhibit expression of provirus of HIV in HIV infected cells and inhibit chronic infections. It would be particularly desirable to have a new therapy that can be used to treat cells already infected, by means of inhibiting expression of provirus, or keep provirus dormant within infected cells.

SUMMARY OF THE INVENTION

We have now discovered that compounds of the following formula I are inhibitory on expression of provirus of HIV-1 and thus are useful for treating cells that have been acutely or chronically infected by immunodeficiency viruses, such as HIV:



I

wherein R and R_1 are each independently selected from the group consisting of hydrogen, substituted and unsubstituted alkyl, substituted and unsubstituted alkenyl, substituted and unsubstituted aryl, and substituted and unsubstituted alkoxy. Preferred compounds of formula I include those in which at least one of the substituents R and R_1 is hydrogen and/or at least one of said substituents is allyl. Specifically preferred compounds include β-lapachone (i.e., R and R_1 both being hydrogen), and allyl-β-lapachone, particularly 3-allyl-β-lapachone (i.e., R being allyl and R_1 being hydrogen).

The compounds of formula I can reduce or inhibit expression of genes operably linked to the HIV LTR.

In one embodiment, the compounds of formula I can treat cells including human cells infected acutely and chronically by immunodeficiency viruses, for example HIV, preferably HIV-1, and thus can be used to treat humans infected by HIV. For example, treatment of those diagnosed as having AIDS as well as those having ARC, PGL and those not yet exhibiting such conditions.

These compounds can be used against a different target than the conventional drugs being used to treat humans infected by HIV, e.g., reverse transcriptase inhibitors such as zidovudine (AZT), 2',3'-dideoxyinosine (ddI) and 2',3'-dideoxycytidine (ddC). Using such drugs in combination with the present compounds is anticipated to result in a synergistic result. Similarly, the present compounds should be effective in cells that are resistant to such compounds. For example, compounds of the present invention can be used to block HIV-1 LTR directed expression in AZT resistant cell lines.

The invention also provides pharmaceutical compositions comprising a compound of formula I and a suitable carrier therefor for use in the conditions referred to above.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the inhibitory effects of β-lapachone (varying concentrations) on TNFα and PMA induced HIV LTR directed gene expression.

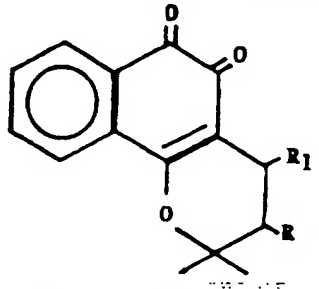
Figure 2 shows kinetics of inhibition on HIV LTR directed cytokine stimulated gene expression by β-lapachone.

Figure 3 shows inhibitory effects of 3-allyl-β-lapachone on HIV LTR directed gene expression.

Figure 4 shows inhibition of HIV-1 replication by β-lapachone in acutely infected human peripheral blood mononuclear cells.

DETAILED DESCRIPTION OF THE INVENTION

We have discovered that compounds of the following formula I can be used to treat cells infected by an immunodeficiency virus, preferably human cells infected with HIV, and thus can be used for treatment in HIV infected individuals:



I

wherein R and R_1 are each independently selected from the group consisting of hydrogen, substituted and unsubstituted aryl, substituted and unsubstituted alkenyl, substituted and unsubstituted alkyl and substituted and unsubstituted alkoxy. The alkyl groups preferably have from 1 to about 15 carbon

atoms, more preferably from 1 to about 10 carbon atoms, still more preferably from 1 to about 6 carbon atoms. As used herein, the term alkyl unless otherwise modified refers to both cyclic and noncyclic groups, although of course cyclic groups will comprise at least three carbon ring members. Straight or branched chain noncyclic alkyl groups are generally more preferred than cyclic groups. Straight chain alkyl groups are generally more preferred than branched. The alkenyl groups preferably have from 2 to about 15 carbon atoms, more preferably from 2 to about 10 carbon atoms, still more preferably from 2 to about 6 carbon atoms. Especially preferred alkenyl groups have 3 carbon atoms (i.e., 1-propenyl or 2-propenyl), with the allyl moiety being particularly preferred. Phenyl and napthyl are generally preferred aryl groups. Alkoxy groups include those alkoxy groups having one or more oxygen linkage and preferably have from 1 to 15 carbon atoms, more preferably from 1 to about 6 carbon atoms. Said substituted R and R_1 groups may be substituted at one or more available positions by one or more suitable groups such as, for example, alkyl groups such as alkyl groups having from 1 to 10 carbon atoms or from 1 to 6 carbon atoms, alkenyl groups such as alkenyl groups having from 2 to 10 carbon atoms or 2 to 6 carbon atoms, aryl groups having from six to ten carbon atoms, halogen such as fluoro, chloro and bromo, and N, O and S, including heteroalkyl, e.g.. heteroalkyl having one or more of said hetero atom linkages (and thus including alkoxy, aminolakyl and thioalkyl) and from 1 to 10 carbon atoms or from 1 to 6 carbon atoms.

It is believed that the compounds of formula I provide effective therapy of chronically infected cells (i.e. cells infected by a virus which is an immunodeficiency virus such as FIV, SIV, HIV, etc.) as evidenced by a reduction in, preferably a complete repression of, HIV LTR directed gene expression. Thus, in an HIV infected cell addition of an effective amount of a compound of formula I will reduce the expression of a gene

operably linked to the HIV LTR. Preferably the gene is operably linked to an HIV-1 LTR. As used herein, the term operably linked means that the gene is under the control of the HIV LTR and positioned in a nucleotide sequence to accomplish this. Typically, the gene is downstream of the LTR, which acts as a promoter. Preferably, the gene corresponds to a viral gene such as the HIV env gene, HIV tat gene, HIV rev gene, etc.

Hence, in one preferred embodiment the present invention can be used in treating those diagnosed as having AIDS as well as those having ARC, PGL and those seropositive but asymptomatic patients. For example, as a preventative, it can also be used prophylactically as a preventative for high risk individuals.

Compounds of formula I can be used to treat cells, especially mammalian cells and in particular human cells, infected by an immunodeficiency virus such as HIV infected cells. As a result of treatment with compounds of formula I viral expression is significantly reduced. β-lapachone and allyl-β-lapachone are preferred compounds of formula I.

For example, HIV viral expression can be studied by a number of methods such as looking at the expression of a marker gene, e.g. CAT, Lac Z, etc., operably linked to the HIV LTR, which acts as the promoter. Use of the present compounds such as β-lapachone can significantly reduce expression of such a marker. HIV viral expression is turned on and enhanced by HIV LTR stimulators such as tumor necrosis factor-α (TNFα) or phorbol 12-myristate-13-acetate (PMA). One product of this expression, i.e., tat can further augment such viral expression. Using a marker gene such as Lac Z operably-linked to the HIV LTR in HIV infected cells, the addition of an effective amount of compounds of formula I significantly inhibits expression of this gene product, thereby indicating that HIV expression under the control of the HIV LTR such as HIV

envelope glycoprotein expression has been inhibited if not completely stopped.

P^{24}, a major structural protein (product of gag), has been widely used for monitoring HIV-1 replication in cells and vireamia in individuals. Use of the present compounds such as β-lapachone, at concentrations that do not significantly adversely affect cells, can dramatically reduce HIV-1 replication, e.g., preferably a reduction of more than 25% as determined by P^{24} levels, more preferably a reduction of more than 50%, and still more preferably a reduction of HIV-1 replication of more than 80% as determined by P^{24} levels.

The effective amount used to obtain such a result is at or below micromolar concentrations. Furthermore, the administration of the compounds of the present invention at effective concentrations, which inhibit HIV expression, has not been found to adversely affect cells.

The compounds of the present invention can be administered to HIV infected individuals or to individuals at high risk for HIV infection. For example, those having sexual relations with an HIV infected partner, intravenous drug users, etc. Because of its inhibitory effect, the compounds of the present invention, or pharmaceutical compositions comprising one ore more compounds of formula I can be used prophylactically as a method of prevention for such individuals to minimize their risk. One would administer the compound at an effective amount as set forth below by methodology such as described herein.

Compounds of formula I can readily be made or obtained. (See Pardee, A., et al., Cancer Research, 49, 1-8 (1989)); Schaffner-Sabba, K., et al., Journal of Medicinal Chemistry, 27, no. 8 990-994 (1984); S. Hooker, 58, 1181-1197 (1936). Other Thus, it is believed compounds of formula I will have utility in

inhibiting the progression of an HIV infection and other retroviral infections in cells and in a human, including utility in extending the latency of an HIV infection in humans.

While not wishing to be bound by theory, the absence of cytotoxicity of the compounds of formula I indicates that these compounds affect positive or negative regulators of HIV LTRs, preferably HIV-1 LTR, that are more critical to the retrovirus than the host cell.

Preferably, for inhibiting or reducing the expression of genes operably linked to an HIV LTR, one or more compounds of the invention is administered in an amount sufficient to reduce of the amount of protein expressed by the gene by at least about 25 percent relative to an untreated cell, more preferably an amount sufficient to reduce the amount of protein by at least about 50 percent and still more preferably a reduction of the amount of protein expressed by least about 75 percent relative to an untreated cell.

In general for the treatment of immunodeficiency viral infections, for example an HIV infection, a suitable effective dose of one or more compounds of formula I will be preferably in the range 1 to 5,000 μg per kilogram body weight of recipient per day, more preferably in the range of 10 to 4,000 μg per kilogram body weight per day. The desired dose is suitably administered once or several more sub-doses administered at appropriate intervals throughout the day, or other appropriate schedule. These sub-doses may be administered as unit dosage forms, for example, containing 1 to 2,000 μg, preferably 10 to 1,000 μg per unit dosage form.

Administration of the compounds of the invention may be by any suitable route including oral, rectal, nasal, topical (including buccal and sublingual), vaginal and parenteral

(including subcutaneous, intramuscular, intravenous and intradermal) with oral or parenteral being preferred. It will be appreciated that the preferred route may vary with, for example, the condition and age of the recipient.

The administered ingredients may be used in therapy in conjunction with other medicaments such as reverse transcriptase inhibitors such as dideoxynucleosides, e.g. zidovudine (AZT), 2',3'-dideoxyinosine (ddI) and 2',3'-dideoxycytidine (ddC), TAT antagonists such as Ro 3-3335, Ro 24-7429, protease inhibitors and other agents such as 9-(2-hydroxyethoxymethyl)guanine (acyclovir), interferon, e.g., alpha-interferon, interleukin II, and phosphonoformate (Foscarnet) or in conjunction with other immune modulation agents including bone marrow or lymphocyte transplants or other medications such as levamisol or thymosin which would increase lymphocyte numbers and/or function as is appropriate. Because many of these drugs are directed to different targets, e.g., viral integration, it is anticipated that a synergistic result will be obtained by this combination.

Similarly, the present compounds may be effective when the above-described drugs are not or are no longer effective. For example, compounds of the present invention can be used in cells that are resistant to reverse transcriptase inhibitors such as AZT, ddI, and ddC. For instance, the compounds of formula (I), can be used to block HIV-1 LTR directed LTR expression in such resistant cell lines and for treatment of such resistant strains. For example, the present compounds can block HIV-1 LTR directed expression in an AZT resistant strain of HIV-1. Accordingly, the present invention can be used therapeutically in an individual as that individual develops resistance to drugs that act on different targets such as AZT, ddI, ddC, RO3-3335, etc. It is expected that the present invention can be used for treatment of HIV-1 infected individuals who develop resistance

to any drug that targets a different state in the viral life cycle than the present compounds.

While one or more compounds of formula I may be administered alone, they also may be present as part of a pharmaceutical composition. The compositions of the invention comprise at least one compound of formula I together with one or more acceptable carriers thereof and optionally other therapeutic ingredients, including those therapeutic agents discussed supra. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

The compositions include those suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration. The compositions may conveniently be presented in unit dosage form, e.g., tablets and sustained release capsules, and in liposomes and may be prepared by any methods well known in the art of pharmacy.

Such methods include the step of bringing into association the to be administered ingredients with the carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers, liposomes or finely divided solid carriers or both, and then if necessary shaping the product.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous

liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion or packed in liposomes and as a bolus, etc.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, preservative, surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein.

Compositions suitable for topical administration include lozenges comprising the ingredients in a flavored basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the ingredient to be administered in a suitable liquid carrier.

Compositions suitable for topical administration to the skin may be presented as ointments, creams, gels and pastes comprising one or more compounds of formula I and a pharmaceutically acceptable carrier. A suitable topical delivery system is a transdermal patch containing the ingredient to be administered.

Compositions suitable for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

Compositions suitable for nasal administration wherein the carrier is a solid include a coarse powder having a particle

size, for example, in the range 20 to 500 microns which is administered in the manner in which snuff is taken, i.e., by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable formulations wherein the carrier is a liquid, for administration, as for example, a nasal spray or as nasal drops, include aqueous or oily solutions of the active ingredient.

Compositions suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such carriers as are known in the art to be appropriate.

Compositions suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable for oral administration may include flavoring agents.

All documents mentioned herein are incorporated herein by reference.

The present invention is further illustrated by the following examples. These examples are provided to aid in the understanding of the invention and are not to be construed as limitations thereof.

GENERAL COMMENTS

The following reagents and procedures were employed as specified in the examples.

Virus. HIV-1 was obtained from the culture supernatant of HTLV-III_B-producing H9 (H9/HTLV-III_B) cells. During the exponential phase of growth, cell-free supernatant was harvested, standardized for reverse transcriptase (RT) activity, and frozen in aliquots at -70°C. Clinical isolates of HIV-1 were prepared from patients testing positive for the human immunodeficiency virus, and standardized for RT activity.

Cells. Cell clone 293.27.2 obtained from L.A. Herzenberg (Stanford University) was derived from human embronic kidney epithelial cells, which were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% Fetal Calf Serum (FCS, obtained from Sigma) plus L-glutamine. See Roederer, M., et al., Proc. Natl. Acad. Sci. USA, 87:4884-4888 (1990). This cell clone had been stably transfected with PNAZ, which is an expression construct of lacZ gene driven by HIV-1 LTR. Expression of β-galactosidase can be greatly increased by administration of PMA or TNFα. Human peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Hypaque gradient centrifugation of blood from HIV-seronegative individuals, and cultured in RPMI 1640 supplemented with 20% FCS (Sigma), penicillin, streptomycin, and L-glutamine in the presence of PHA (3 μg/ml). RPMI 8402 cell line, received

from Tushino Ando (Aichi Cancer Research Institute, Nagoya, Japan), is a human T lymphatic cell line. It was grown in RPMI 1640 medium supplemented with 15% FCS and L-glutamine.

Stock Solution. A stock solution was prepared of β-lapachone in dimethylsulfoxide (DMSO) at a 20 mM concentration. Aliquots of the stock solution were stored frozen at -20°C.

Quantitation of HIV-1 LTR directed gene expression. Exponentially growing 293.27.2 cells (L.A. Herzenberg of Stanford University) were plated in 6 well plates at 2 x 10^5 cells per well in 2 ml of growth medium. After 48 hours, the cells were stimulated with 40 u/ml of TNFα (Genzyme, Cambridge, MA) or 2 ng/ml of PMA (Sigma). Various concentrations of β-lapachone was added to the medium at designated times after stimulation with final concentrations of DMSO or ethanol at less than 0.1% (v/v). After 6-8 hours incubation at 37°C, the medium was aspirated, cells were harvested, washed 4 times with PBS, and lysed in lacZ buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$). β-galactosidase activities of cell lysates were quantitated by using ONPG as substrate. See, e.g. Herbomel, et al., Cell, 39:653-662 (1984). Protein concentration was measured. Cell visibility was determined by the colony formation assay after cells were treated as above.

Treatment in acute HIV-1 infection. Peripheral blood mononuclear cells (PBMC), after 72 hours stimulation with 3 μg/ml PHA, were infected with either HTLV-III or a clinical isolate of HIV-1 at 1 reverse transcriptase unit (RTU) per 10 cells. Infection was carried out at 37°C for 2 hours. Then PBMC were washed with PBS to remove free virus and replated at 4.5 X 10^6 cells per well in 2 ml medium in the absence or presence of different concentrations of drugs. The cells were

then continuously exposed to drugs for 6 days. On day 3, 1 ml of medium was removed from each well and replaced with 1 ml of fresh media containing drug at the previous concentration. On day 6, cells and media were harvested. Cell viability was determined by the trypan blue exclusion method and MTT metabolic assays. See Mosman, T., J. Immunological Methods, 65:55-63 (1983). P^{24} levels in the culture supernatant were quantitated by ELISA assay with HIV-1 P^{24} Antigen Kinetics Assay Kit (Coulter, Hialeah, FL) used according to the manufacturer's protocol. RNA of HIV-1 reverse transcriptase (RT RNA) was assayed with HIV-1 RNA Detection Kit (GeneTrak, Framingham, MA) according to manufacturer's instructions. Briefly, the total cellular RNA was prepared, dot blotted onto a nitrocellulose membrane, and hybridized with ^{32}P labeled probe for RT RNA.

EXAMPLE 1

The effects of β-lapachone on HIV LTR directed cytokine enhanced gene expression (LacZ) was examined as described below, with the results depicted in Figure 1. A model HIV provirus transfected cell line, 293.27.2 (see General Comments above), was plated in 6 well plates at 2 X 10^5 cells/well in 2 ml of growth medium (DMEM). TNFα (40 u/ml) and PMA (2 ng/ml) together with various concentrations of β-lapachone as indicated in Figure 1 were added to culture media 48 hours after the cells were plated. Six hours after addition of the drug, the cells were harvested and β-galactosidase activity was measured with ONPG as the substrate by the methodology described in Herbomel, et al., Cell, 39:653-662 (1984). Enzymatic activity was expressed as percentage of maximum expression which is referred to that in drug untreated sample (taken as 100). The experiments were repeated on three independent occasions. For cell survival analysis, 293.27.2 cells were treated similarly as above. After treatment with different concentrations of β-lapachone for 6 hours, cells were

trypsinized and replaced in triplicate at 100, 1,000, 10,000 cells per 60 mm plate. After 7 day incubation, cell cloning (more than 50 cells) were counted. Plating efficiency was around 20%. Results are depicted in Figure 1 where each data point is expressed as percent survival of control, i.e. drug untreated plates (taken as 100).

As depicted in Figure 1, β-lapachone exhibited strong inhibition of expression of HIV LTR in the absence of a decrease in cell survival. β-lapachone provided inhibition against stimulation by PMA at an IC_{50} of 0.3 μM, and inhibition against stimulation by TNFα at 0.7 μM. As shown in Figure 1, β-lapachone can reduce HIV LTR activity to near zero, indicating its inhibition of basal activity. These results suggest that the compounds of formula I inhibit LTR-activation of HIV-1 provirus.

EXAMPLE 2

Cells were plated as described in Example 1 above. TNFα (40 u/ml) was added to culture media forty eight hours after the cells were plated or PMA (2 ng/ml). β-lapachone at a concentration of 2 μM was added to culture media with TNFα. β-galactosidase was assayed as described in Example 1 above. Experiments were repeated twice independently. Results are depicted in Figure 2 where each data point represents an average of triplicate culture walls.

EXAMPLE 3

The same procedures described in Example 2 above was repeated using 3-allyl-β-lapachone in place of β-lapachone with the results depicted in Figure 3.

EXAMPLE 4

The effect on HIV-1 replication in acutely infected PBMC by β-lapachone was examined as described below with the results

depicted in Figure 4. Human PBMC were infected with HTLV-IIIB at 37°C for 2 hours. After stimulation with PHA (3 μg/ml) for 72 hours, free virus were removed by wash with PBS. Different concentrations of β-lapachone was added to the culture media in less than 0.1% (v/v) of DMSO. P^{24} levels in culture supernatant were determined as described in the General Comments above. Cell viability was ascertained by trypan blue exclusion. As shown in Figure 4, a dose dependent decline in HIV-1 replication was evident for β-lapachone in the absence of significant toxicity. The therapeutic index (defined herein as the ratio of the 50% cell growth inhibitory concentration to 50% virus inhibitory concentration) was estimated to be 5.0.

EXAMPLE 5

Inhibition of HIV-1 expression in chronically infected human T cell lines. RPMI 8402 were infected with HTLV-III. β-Lapachone was added to the culture media of the chronically infected cells (2×10^5 cell/ml). The final concentrations of DMSO in media were kept at less than 0.1%. Three and six days after addition of drugs, P^{24} levels in the culture supernatant were determined as described in the General Comments above. Cell viability was determined by MTT as described in the General Comments. Results are shown in the Table below.

TABLE

Inhibition of HIV-1 replication in chronically infected human T lymphocytes by β-Lapachone*

β-lapachone concentration (μM)	inhibition of P^{24} (%)	cell viability (%)
0	0	100
0.625	18	100
2.5	60	100

* RPMI 8402 cell line was used.

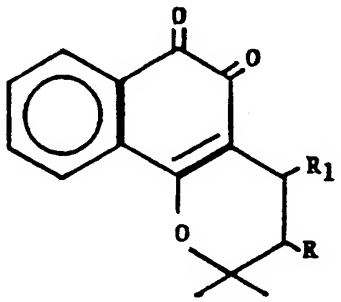
EXAMPLE 6

In a chronically infected T-lymphocyte line and the promyelocytic cell line OM10.1, inhibition of expression of P^{24} antigen was seen at IC_{50} - 0.057 μM following treatment with β-lapachone whereas AZT and ddI had little effect. The cell line OM10.1 contains one copy of HIV-1 cell integrated into its genome and continually produces a low level of HIV-1 proteins. This cell line was kindly supplied by NIH-AIDS Research and Reference Reagent Program.

This invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modification and improvements within the spirit and scope of the invention.

What is claimed is:

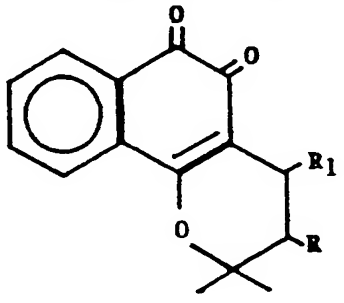
1. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an effective antiviral treatment amount a compound of the formula I:



I

wherein R and R_1 are each independently selected from the group consisting of hydrogen, substituted and unsubstituted alkyl, substituted and unsubstituted alkenyl, substituted and unsubstituted aryl, and substituted and unsubstituted alkoxy, adapted for treating a mammal having an immunodeficiency virus.

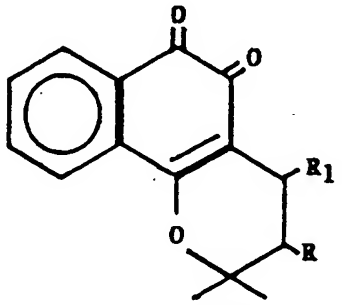
2. A method of inhibiting or reducing the expression of genes operably linked to an LTR of an immunodeficiency virus which comprises administering an effective gene expression reduction amount of a compound of the formula I:



I

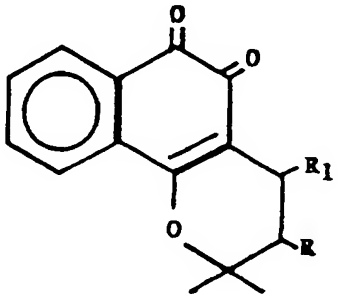
wherein R and R_1 are each independently selected from the group consisting of hydrogen, substituted and unsubstituted alkyl, substituted and unsubstituted alkenyl, substituted and unsubstituted aryl, and substituted and unsubstituted alkoxy.

3. A method for treating cells infected with a virus capable of causing an immunodeficiency disease in a human, comprising administering to the cells an effective antiviral treatment amount of a compound of the following formula I:



wherein R and R_1 are each independently selected from the group consisting of hydrogen, substituted and unsubstituted alkyl, substituted and unsubstituted alkenyl, substituted and unsubstituted aryl, and substituted and unsubstituted alkoxy.

4. A method of treating a human having an immunodeficiency disease comprising administering to said human an effective immunodeficiency disease treatment amount of a compound of the following formula I:



I

wherein R and R_1 are each independently selected from the group consisting of hydrogen, substituted and unsubstituted alkyl, substituted and unsubstituted alkenyl, substituted and unsubstituted aryl, and substituted and unsubstituted alkoxy.

5. The immunodeficiency virus of claims 1, 2, 3 or 4 which is resistant to a reverse transcriptive inhibitor.

6. The reverse transcriptive inhyibitor described in claim 5, which is selected from the group consisting of zidovudine (AZT), 2',3'-dideoxyinesine (ddI) and 2',3'-dideoxyctidine (ddC).

7. The reverse transcriptive inhibitor described in claim 6 which is AZT.

8. The method of claim 2 wherein the LTR is an HIV LTR.

9. The method of claim 2 wherein the LTR is an HIV-1 LTR.

10. The compound described in claims 1, 2, 3 or 4, where at least one of R and R_1 is hydrogen.

11. The compound described in claims 1, 2, 3 or 4, where at least one of R and R_1 is alkenyl.

12. The compound described in claims 1, 2, 3 or 4, where at least one of R and R_1 is allyl.

13. The compound described in claims 1, 2, 3 or 4 where the compound of formula I is selected from the group consisting of β-lapachone and allyl-β-lapachone.

14. The compound described in claim 13, where the compound of formula I is 3-allyl-β-lapachone.

15. The method of claim 3 where the cells are mammalian cells.

16. The method of claim 3 where the cells are human cells.

17. The immunodeficiency virus described in claims 1, 2, 3, 4 or 5 where the virus is capable of causing in the human acquired immune deficiency syndrome or an acquired immune deficiency syndrome related complex.

18. The virus described in claim 17 where the virus is HIV.

19. The virus described in claim 18 where the virus is HIV-1.

20. The method of claim 4 in which the human has antibodies to the HIV virus.

21. The method of claim 4 in which the human has antibodies to the HIV-1 virus.

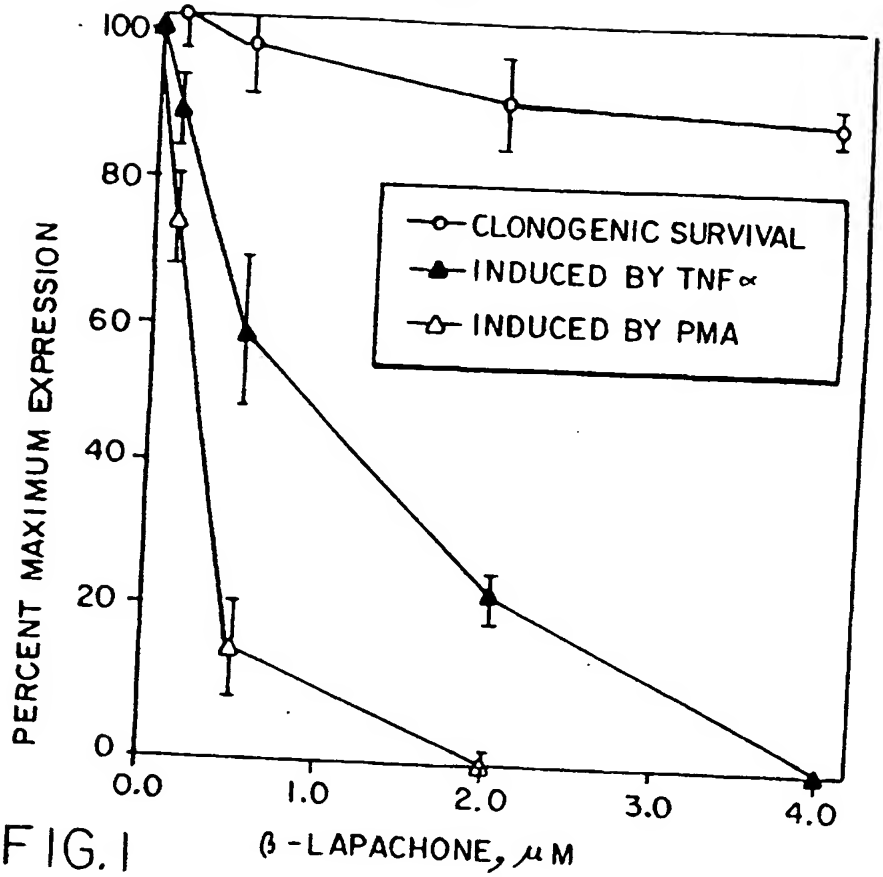

100
80
60
40
20
0
PERCENT MAXIMUM EXPRESSION
CLONOGENIC SURVIVAL
INDUCED BY TNFα
INDUCED BY PMA
0.0
1.0
2.0
3.0
4.0
β-LAPACHONE, μM

FIG. 1

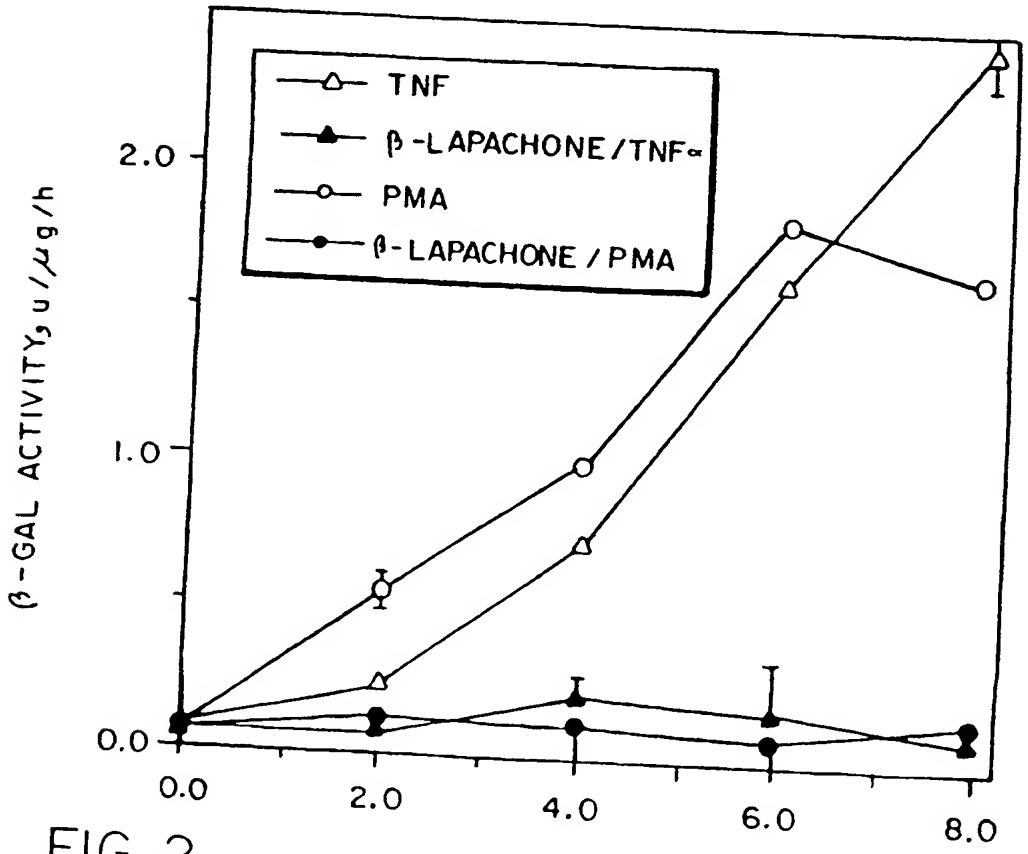

TNF
β-LAPACHONE/TNFα
PMA
β-LAPACHONE/PMA
2.0
1.0
0.0
β-GAL ACTIVITY, u/μg/h
0.0
2.0
4.0
6.0
8.0

FIG. 2

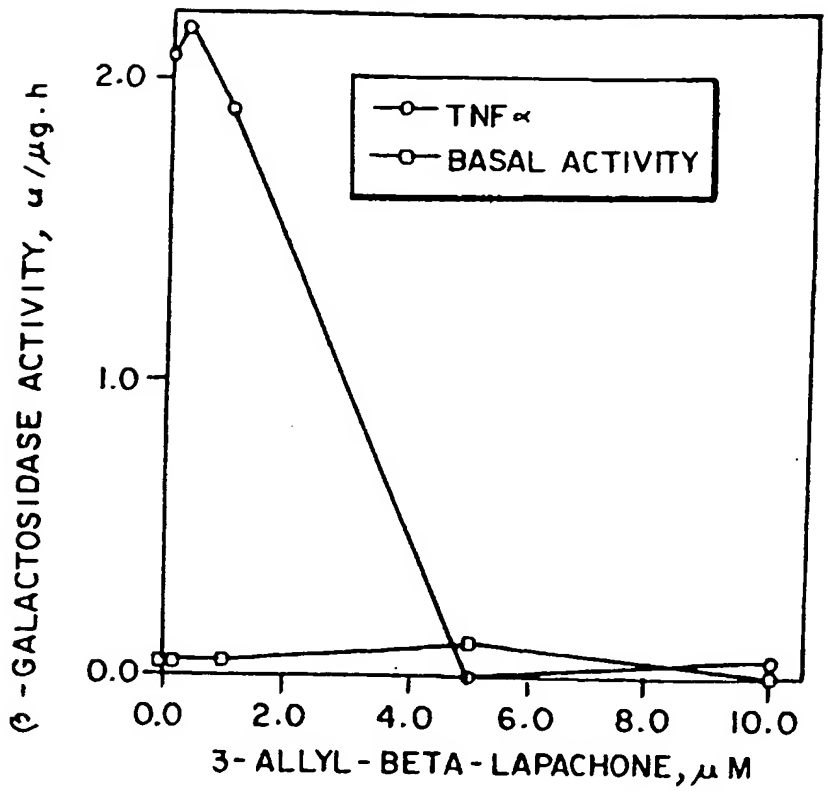

TNFα
BASAL ACTIVITY
β-GALACTOSIDASE ACTIVITY, u/μg.h
2.0
1.0
0.0
0.0
2.0
4.0
6.0
8.0
10.0
3-ALLYL-BETA-LAPACHONE, μM

FIG. 3

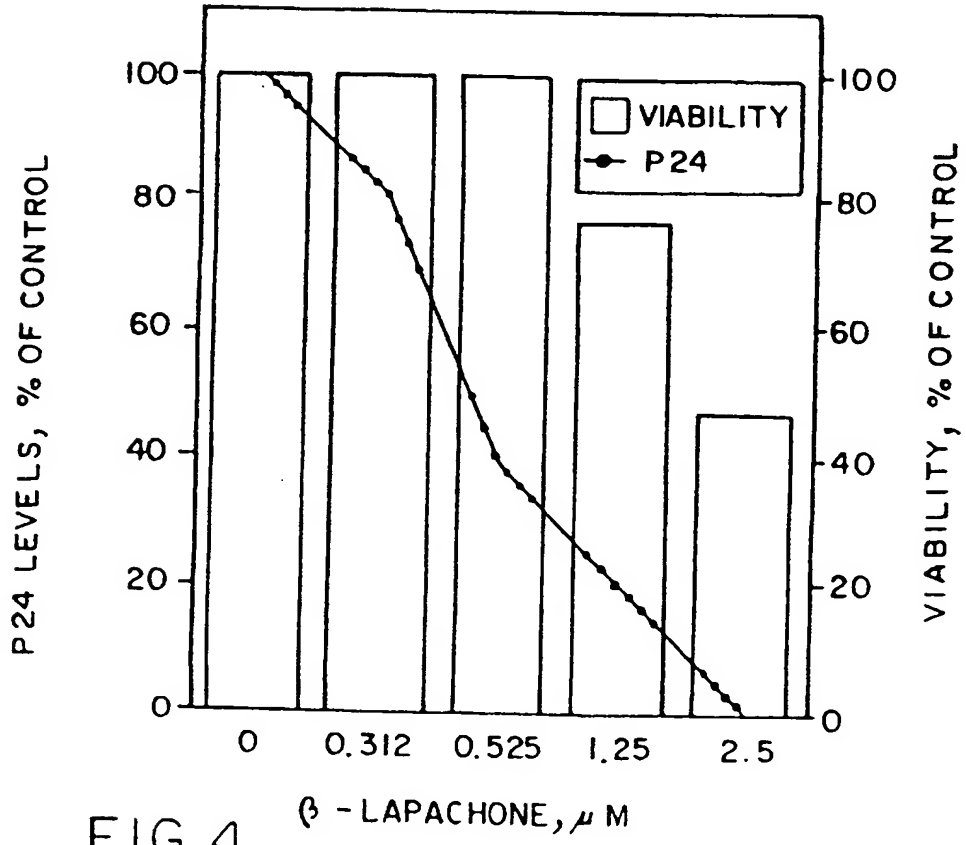

VIABILITY
P24
P24 LEVELS, % OF CONTROL
VIABILITY, % OF CONTROL
100
80
60
40
20
0
0
0.312
0.525
1.25
2.5
β-LAPACHONE, μM

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US93/07878

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 31/35

US CL :514/455

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/455

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN - Structure, Antiviral methods of use AIDS, HIV

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Chemical Abstracts, Volume 101, No. 7, 13 August 1984, issued (Columbus, Ohio, U.S.A.), "Schaffner-Sabba et al, "Beta-Lapachone: synthesis and activities in tumor models". See the abstract no. 54755x. Chem. biol. Res. Lab., Ciba-Geigy Ltd., Basle, Switz.), J. Med. Chem., 1984, vol. 27, no. 8, pp. 990-994.	1-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

12 NOVEMBER 1993

Date of mailing of the international search report

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Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Chemical Abstracts, Volume 88, no. 20, issued 13 November 1978 (Columbus, Ohio, U.S.A.), Schurech et a, "Beta-Lapachone, an inhibitor for oncornavirus referse transcriptase and eukaryotic DNA polymerase-alpha. Inhibitory effect, thiol dependency and specificity". (Pharm. Div., Ciba-Geigy Ltd., Basel, Switz.) See abstract no. 165933, Eur. J. Biohcem. 1978, vol. 84, no. 1, pp. 197-205.	1-21